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COMMUNICATIONS

11-fold Symmetry of the *trp* RNA-binding Attenuation Protein (TRAP) from *Bacillus subtilis* Determined by X-ray Analysis

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The *trp* RNA-binding attenuation protein (TRAP) of *Bacillus subtilis* has been crystallized and examined by crystallography using X-ray synchrotron radiation diffraction data. Crystals of TRAP complexed with L-tryptophan belong to space group $C2$ with $a = 156.8$ Å, $b = 114.05$ Å, $c = 105.9$ Å, $\beta = 118.2°$. Crystals of a potential heavy-atom derivative of TRAP complexed with 5-bromo-L-tryptophan grow in the same space group with similar cell dimensions. X-ray data for the native crystals and for the derivative have been collected to 2.9 Å and 2.2 Å resolution, respectively. Peaks in the self-rotation function and in the Patterson synthesis could only be explained by two 11-subunit oligomers (each formed by an 11-fold axis of symmetry) in the asymmetric unit lying with the 11-fold rotation axes parallel to each other. The consequence is that the TRAP molecule has 11-fold symmetry and contains 11 subunits.

Keywords: *trp* attenuation protein; RNA-binding protein; crystallization; X-ray analysis

Transcription of the *Bacillus subtilis* tryptophan (*trpEDCFBA*) operon is negatively regulated by an RNA-binding protein, *trp* RNA-binding attenuation protein (TRAP†) (Shimotsu *et al.*, 1986; Babitzke & Yanofsky, 1993; Otridge & Gollnick, 1993; Gollnick, 1994). This protein is the product of the *mtrB* gene, which encodes a 75 amino acid polypeptide of M_r 8328 (Gollnick *et al.*, 1990; Babitzke *et al.*, 1992). TRAP functions as an oligomer of identical subunits by binding to the leader transcript of the *trp* operon and influencing the secondary structure of the RNA (Babitzke & Yanofsky, 1993; Otridge & Gollnick, 1993). The 204 nucleotide *trp* leader transcript is capable of forming several alternative hairpin structures (Shimotsu *et al.*, 1986). One of these (C:D) forms a transcription terminator, whereas a second hairpin (A:B) is capable of forming just upstream of the terminator. Formation of the upstream structure interferes with formation of the terminator, hence A:B is designated the antiterminator. The mechanism for selecting between these structures involves the RNA-binding protein TRAP (Gollnick, 1994). In the presence of tryptophan, TRAP is activated to bind to the *trp* leader RNA and induce formation of the terminator, turning off expression of the operon. When cells are grown in the absence of tryptophan, TRAP does not bind to the leader RNA and the antiterminator forms, allowing expression of the operon.

Purified TRAP binds specifically to *trp* leader RNA *in vitro* in a tryptophan-dependent manner, and does not bind to analogous DNA sequences (Otridge & Gollnick, 1993). Moreover, addition of purified TRAP increased transcription termination in an *in vitro* transcription system using *B. subtilis* σ^{43} RNA polymerase (Babitzke & Yanofsky, 1993). The amino acid sequence of TRAP is not highly homologous with other known RNA-binding proteins (Gollnick *et al.*, 1990). Kuroda *et al.* (1988) proposed that TRAP recognizes a ten base sequence repeated twice in the leader transcript. However, recent studies by Babitzke *et al.* (1994) indicate that TRAP recognizes a trinucleotide motif (G/UAG), which is repeated 11 times in the *B. subtilis trp* leader region.

Previous studies have been unclear with regard to the number of subunits in the TRAP molecule. Size-exclusion chromatography yielded single peaks corresponding to a molecular weight of approximately 50 to 60 kDa (six to eight subunits per

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† Abbreviations used: TRAP, *trp* RNA-binding attenuation protein; PMSF, phenylmethylsulfonyl fluoride; MPD, 2-methyl-2,4-pentanediol.

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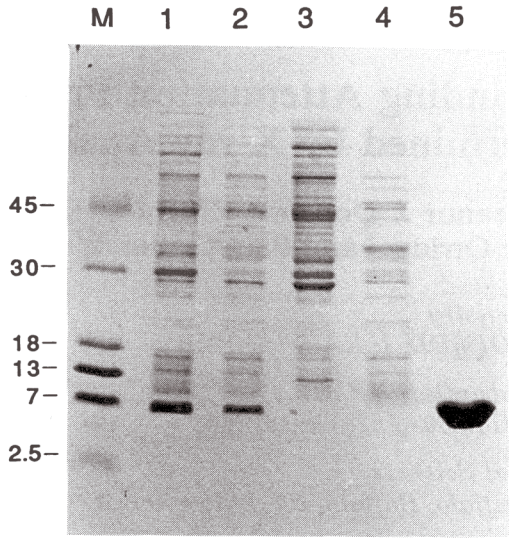

Figure 1. SDS-PAGE (9% to 18% (w/v) gradient) of TRAP purification by phenyl agarose. Lanes: 1, soluble protein extract from *Escherichia coli* SG62052/pGP1-2/pTZmtrB (40 μg); 2, 50% ammonium sulfate supernatant (30 μg); 3, 15% ammonium sulfate wash (40 μg); 4, buffer wash (20 μg); 5, TRAP eluted with 25% ethylene glycol (40 μg). Lane M, molecular size standards (in kDa) are indicated. The gel was stained with Coomassie brilliant blue.

molecule: Otridge & Gollnick, 1993; Babitzke & Yanofsky, 1993), whereas mass spectrometry of cross-linked TRAP showed peaks corresponding to between 11 and 13 subunits (Babitzke *et al.*, 1994). To determine the quaternary structure of the TRAP molecule and to aid in understanding the mechanism by which TRAP is activated by L-tryptophan to recognize a specific RNA target, we have undertaken X-ray crystallographic studies of this protein. Here we report deduction of the symmetry of the TRAP oligomer on the basis of crystallization and X-ray data. These studies were performed for complexes of TRAP with L-tryptophan and with 5-bromo-L-tryptophan, where the latter complex is a potential derivative for structure solution by isomorphous replacement.

Table 1
Purification of TRAP from Escherichia coli *cell extract*

	Total volume (ml)	Concentration of protein (mg/ml)	Total protein (mg)
Cell extract	12	12.75	177.0
Phenyl column charge 50% $(NH_4)_2SO_4$ cut	19	4.0	76.0
Phenyl column flow through	139	0.085	11.80
Phenyl column 15% $(NH_4)_2SO_4$ in 0.025 M Tris · HCl (pH 8.5), wash	84	0.380	31.92
Phenyl column 0.025 M Tris · HCl (pH 8.5), wash	120	0.150	18.0
Econo-Pac Q cartridge pool	6	2.56	15.36

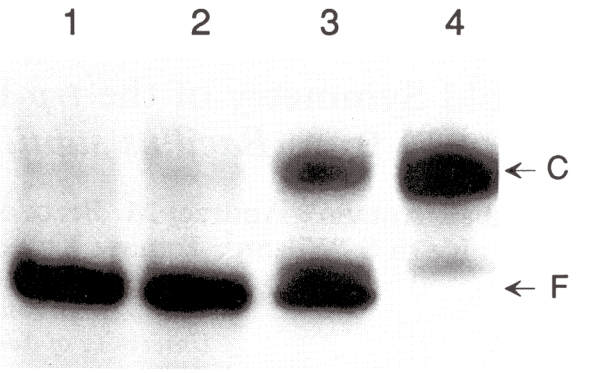

Figure 2. RNA mobility shift assay of TRAP interaction with *B. subtilis trp* leader RNA transcript with either 5-bromo-L-tryptophan or L-tryptophan. ^{32}P-labelled *in vitro* transcribed RNA (– 2 to + 138) was incubated with 80 ng of TRAP and electrophoresed on a 6% (w/v) native polyacrylamide gel as described by Otridge & Gollnick (1993). Lanes: 1, RNA in the absence of protein; 2, RNA with TRAP; 3, TRAP with 350 μM 5-bromo-L-tryptophan; 4, TRAP with 240 μM L-tryptophan. F, free RNA; C, complexed RNA and TRAP.

We also report a new simple method to purify large amounts of TRAP from overexpressing *Escherichia coli* SG62052(pGP1-2) (Tabor & Richardson, 1985) transformed with pTZ18mtrB (Otridge & Gollnick, 1993), which greatly accelerated various crystallization trials. TRAP is expressed to approximately 10% of the soluble protein in these cells (Figure 1). Cells were harvested by centrifugation, resuspended in lysate buffer (100 mM K_2HPO_4, 50 mM KCl, 1 mM EDTA, 160 μg PMSF/ml, pH 6.0) and broken in a French pressure cell at 1200 lb/in^2. The lysate was cleared at 30,000 ***g*** for 30 minutes and $(NH_4)_2SO_4$ was added to 50% saturation. The precipitate was removed by centrifugation at 30,000 ***g*** for 30 minutes and the supernatant was applied directly to a column of phenyl agarose (Sigma) equilibrated with 25 mM Tris · HCl (pH 8.5), 50% saturated $(NH_4)_2SO_4$. The column was washed successively with 25 mM Tris · HCl (pH 8.5) buffer 50% saturated with $(NH_4)_2SO_4$, followed by the same buffer 15% saturated with $(NH_4)_2SO_4$, and finally buffer alone. TRAP was then eluted from the column with 25% (v/v) ethylene glycol in 25 mM Tris · HCl (pH 8.5). TRAP-containing fractions were applied to an Econo-Pac Q ion exchange column (BioRad). TRAP was eluted from this column using a NaCl gradient (0 to 1.0 M) in Tris · HCl (pH 8.0). TRAP purified by this method was judged to be greater than 95% pure based on Coomassie blue-stained SDS-polyacrylamide gels (Figure 1). Yields were 10 to 20 mg of TRAP/liter of cell culture (Table 1).

L-Tryptophan binds to TRAP in a highly cooperative fashion with a binding constant of approximately 12 μM as determined by equilibrium dialysis (T. Smith & P. Gollnick, unpublished observations). Several observations indicate 5-bromo-L-tryptophan also binds to TRAP. 5-Bromo-L-tryptophan activated TRAP to bind *trp* leader RNA in a

Table 2
Statistics of the synchrotron diffraction data

Data set	Resolution (Å)	R_{merge}†	Completeness (%)	Redundancy‡
L-Tryptophan	2.9–15	0.094	94.7	3.01
5-Bromo-L-tryptophan	2.2–20	0.095	97.7	3.96

90.1% of the 5-bromo-L-tryptophan data contained both, F^+ and F^- anomalous pair measurements. The mean isomorphous difference between measurements of the same reflections for 5-bromo-L-tryptophan and L-tryptophan data is 22.4% in the resolution shell from 10.0 to 2.9 Å ($R_{iso} = (\Sigma||F_{ph}| - |F_p||/\Sigma(|F_{ph}| + |F_p|)/2) \times 100$.

† The value of the merging R factor between equivalent measurements of the same reflection: ($R_I = \Sigma|I - \langle I\rangle|/\Sigma I$).

‡ The average number of observations of the same reflection.

mobility shift assay in a similar manner as L-tryptophan (Figure 2). Furthermore, 5-bromo-L-tryptophan inhibited growth of *mtrB*$^+$ *B. subtilis* and not of *mtrB*$^-$ cells. This effect has also been seen with 5-methyl-L-tryptophan (Hoch *et al.*, 1971) and 5-fluoro-L-tryptophan, and is due to these tryptophan analogs activating TRAP to repress expression of the *trp* operon but not functioning in protein synthesis. This results in *mtr*$^+$ cells starving for tryptophan, whereas *mtr*$^-$ cells continue to synthesize tryptophan allowing them to survive. These observations suggest that 5-bromo-L-tryptophan also activates TRAP to repress expression of the *trp* operon *in vivo*.

Crystallization of TRAP was performed at 20°C using hanging drop vapor diffusion. Prior to crystallization the purified protein was dialyzed against 0.1 M sodium phosphate (pH 6.0) and concentrated using a 30 K ultrafiltration membrane (Filtron) to 20 mg/ml. The reservoir contained 0.1 M sodium phosphate (pH 6.0), 30 mM of either L-tryptophan or 5-bromo-L-tryptophan and a precipitant. For crystallization, 1 μl aliquots of the protein solution were mixed with an equal volume of reservoir solution. Crystals have been obtained with various poly(ethylene) glycols (2000, 4000, 8000) or monomethylether poly(ethylene) glycols (2000 or 5000) as precipitants. With monomethyl ether polyethylene glycols, which have been shown to be useful for crystal growth (Brzozowski, 1993), crystals grew to larger dimensions than with the commonly used poly(ethylene) glycols. Crystals of about 0.1 mm × 0.1 mm × 0.1 mm grew in the presence of 4 to 5% (w/v) polyethylene glycol. Crystals appeared in four to six hours as rhombic prisms and attained maximal size within a few days. Crystals similar in size and shape were obtained with 9% (v/v) MPD, 9% (v/v) ethanol or isopropanol, 16% (v/v) acetone, 12% (v/v) 1,4 dioxane, or 18% (v/v) butane-1,4-diol as precipitant. Without L-tryptophan or its analog, 5-bromo-L-tryptophan, we were not able to obtain crystals in the pH range of pH 5.0 to 9.0 using various organic and inorganic precipitants.

The crystals were characterized on a Rigaku RAXIS IIC imaging plate detector, mounted on a RU200 Rigaku rotating anode X-ray generator operating at 50 kV and 100 mA, utilizing a graphite monochromator and 0.3 mm collimator. From each crystal, a single diffraction pattern with rotation of the crystal by one degree was recorded. The cell dimensions and space group were determined using the program DENZO (Z. Otwinowski, Yale University).

The largest crystals of TRAP, with dimensions of 0.2 mm × 0.2 mm × 0.2 mm, were grown with 5-bromo-L-tryptophan as the ligand, using 2000 monomethyl ether poly(ethylene) glycol. These crystals diffracted to 2.0 to 2.2 Å resolution and belong to space group $C2$ with $a = 157.1$ Å, $b = 114.7$ Å, $c = 106.3$ Å, $\beta = 117.7°$. In contrast, crystals obtained under analogous conditions but with L-tryptophan as the ligand gave highly split diffraction, presumably caused by a polycrystalline nature of these crystals. This effect was seen when polyethylene glycol, MPD, 1,4 dioxane or butane-1,4-diol were used as precipitants. However, when ethanol or isopropanol were used the crystals grew in space group $C2$ with cell dimensions very close to those of crystals obtained with 5-bromo-L-tryptophan ($a = 156.8$ Å, $b = 114.05$ Å, $c = 105.9$ Å, $\beta = 118.2°$). The largest crystals obtained with L-tryptophan were 0.15 mm × 0.15 mm × 0.15 mm, and the high resolution diffraction limit was 2.7 Å.

Diffraction data from both complexes of TRAP were collected using synchrotron radiation on EMBL/Hamburg beamlines with a MAR Research image-plate scanner as the detector. Data to 2.2 Å resolution from the 5-bromo-L-tryptophan complex were obtained from a single crystal on beamline X31 at a wavelength of 0.91 Å, which is close to the Br absorption edge from the high energy side of the spectrum. Data from crystals of TRAP complexed with L-tryptophan were collected from three crystals to a resolution of 2.9 Å on wiggler beamline BW7B ($\lambda = 0.90$ Å). The data sets were processed using the program DENZO (Z. Otwinowski, Yale University). The statistics of the data sets are summarized in Table 2.

The internal symmetry of the TRAP molecule was deduced from the self-rotation function $R(\Phi, \Psi, K)$ (Crowther, 1972) using the CCP4 program package (SERC Daresbury Laboratory, England). Calculations were performed with a radius of integration sphere of 20 Å using data from crystals of TRAP complexed with 5-bromo-L-tryptophan in the resolution shell 3.5 to 10.0 Å. The self rotation function showed 11 peaks with $\kappa = 180°$ (Figure 3A) spaced

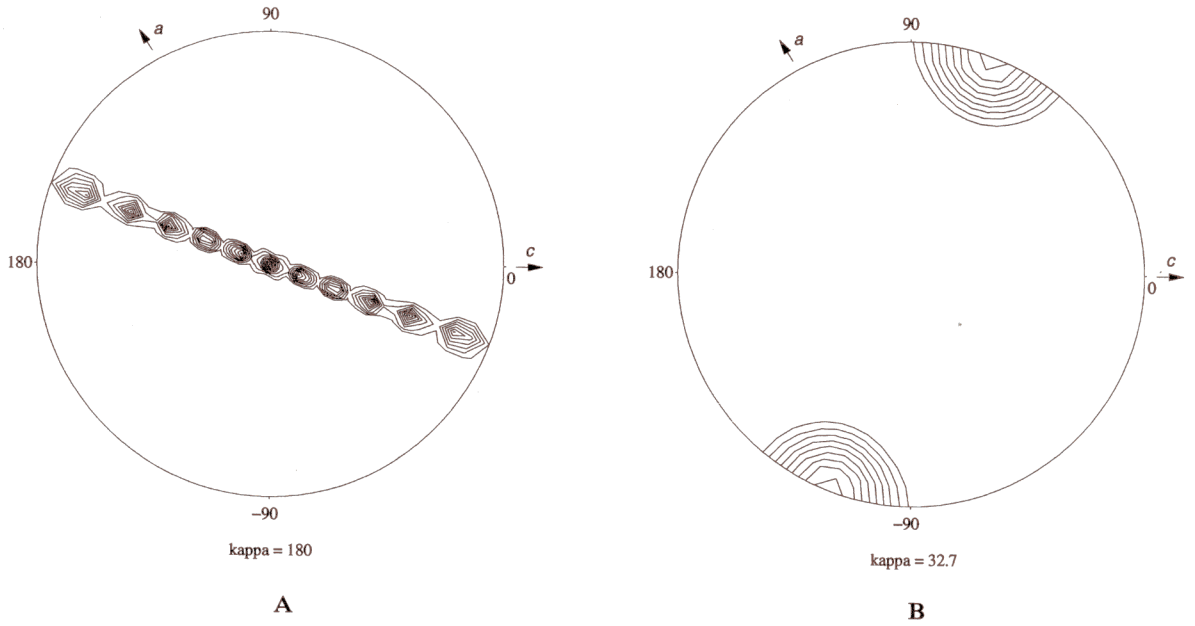

Figure 3. Stereographic projections of self-rotation function with crystallographic 2-fold axis perpendicular to the plane of the Figure. The ϕ angle varies from 0° to ±180° around the plot while the ψ angle varies from 0° at the center to 90° at the outer ring. The crystallographic *b* axis points upwards along $\psi = 0°$, the *a* axis is along $\phi = 118°$, $\psi = 90°$ and the *c* axis along $\phi = 0°$, $\psi = 90°$. A, Section with $\kappa = 180°$; B, section with $\kappa = 32.7°$. Contouring starts at 2.0 σ level and the interval is 0.5 σ.

from each other by 32.7° along ψ and a peak at $\kappa = 32.7°$, $\psi = 90°$ (Figure 3B). These peaks in the self-rotation function remained present when the radius of integration was varied from 15 to 40 Å and are the only peaks higher than the 2.0 σ level. The same peaks also appeared in the self-rotation function calculated from native, L-tryptophan, data sets. The peak at $\kappa = 32.7°$, $\psi = 90°$ could be generated by a molecule containing 11 subunits arranged in a ring with an 11-fold axis of symmetry lying perpendicular to the crystallographic *b* axis. This arrangement in conjunction with the crystallographic dyad would generate 11 peaks with $\kappa = 180°$ separated from each other by 32.7° along ψ. The constellation of peaks in the self-rotation function could be produced either by one such oligomer or by two oligomers with parallel internal 11-fold axes of symmetry. To distinguish between these two cases we calculated Patterson maps (Figure 4), which showed a peak at (1/2, 0, 1/2) with a height of about 40% of the origin peak. Hence there must be two 11-subunit oligomers in the asymmetric unit lying in the same orientation. The (1/2, 0, 1/2) translation together with the crystallographic dyad (which is oriented parallel to the *b* axis in space group *C*2) leads to formation of a non-crystallographic dyad, which lies parallel to the crystallographic one.

Further evidence for two TRAP oligomers per asymmetric unit comes from calculation of the solvent content. Two 11-subunit molecules of TRAP per asymmetric part of the crystal gives a specific volume (Matthews, 1968), V_M, of 2.28 Å^3/Da and a solvent content of 45.7%. One 11-subunit oligomer per asymmetric unit would result in a specific volume of 4.57 Å^3/Da and solvent content of 72.9%, which are unusual values for protein crystals.

The peak corresponding to the 11-fold symmetry axis lies at $\phi = 70°$, $\psi = 90°$ (Figure 3B). Thus, the pair of TRAP molecules lies in the unit cell with their 11-fold axes oriented at an angle of 48° to the *a* axis. In addition, the (1/2, 0, 1/2) translation between the pair of oligomers requires that their 11-fold axes are not just parallel, but are lying in the same plane perpendicular to the *b* axis (for example in plane $Y = 0$, as the origin in the *C*2 space group is not fixed along *Y*). Figure 5 represents the arrangement of TRAP oligomers in the unit cell, where the A and B oligomers are related by a (1/2, 0, 1/2) translation, as are the A' and B' oligomers. The A and A' as well as the B and B' oligomers are related by crystallographic

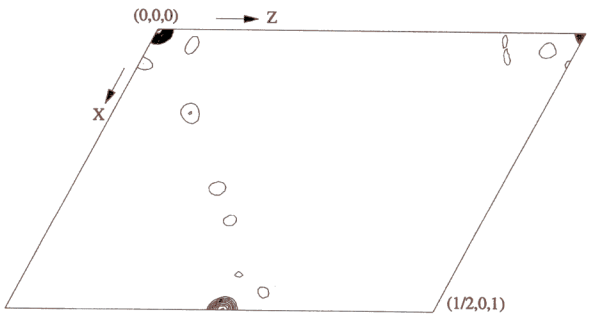

Figure 4. Section $Y = 0$ of the Patterson synthesis calculated from the 5-bromo-L-tryptophan data set in a resolution shell 6.0 to 10.0 Å. The crystallographic *a* axis lies along *X*, and the *c* axis lies along *Z*. *X* varies from 0 to 1/2, *Z* varies from 0 to 1. Contouring starts at the 3.0 σ level and the interval is 3 σ.

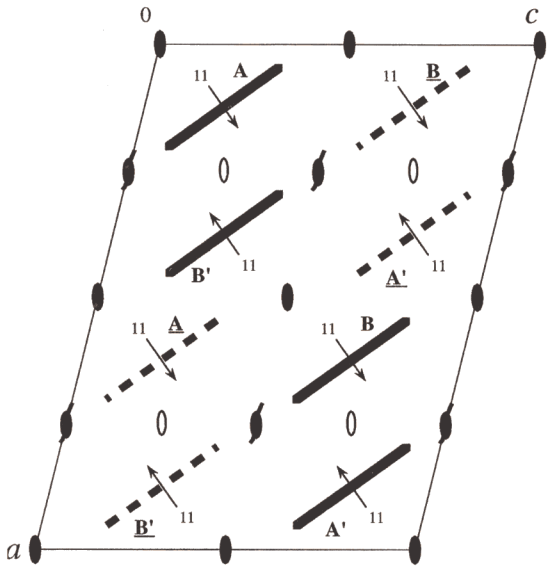

Figure 5. Schematic plot of a possible packing arrangement of the TRAP oligomers in the *C*2 unit cell with crystallographic 2-fold axes perpendicular to the plane of the Figure. A, A', B and B' oligomers (shown in bold) are oriented with their 11-fold axes of symmetry (designated by arrows) lying in the same plane. The other 4 oligomers (shown by broken lines) are designated by underlined letters and are related to the first 4 oligomers by the crystallographic (1/2, 0, 1/2) translation. Black lenses represent crystallographic 2-fold axes, empty lenses designate non-crystallographic 2-fold axes.

symmetry. The 11-fold axes of the A, A', B and B' oligomers may coincide.

Data from laser-light-scattering experiments to determine the molecular mass of the native molecule in solution are in agreement with the conclusion that TRAP exists as an 11-subunit molecule. Duplicate analyses using DAWN triple-angle light scattering detector (Wyatt Technology Corp.) with a He–Ne laser operating at 633 nm, yielded molecular masses of 94,400 and 95,300. These values correspond to 11.33 and 11.44 subunits per molecule, respectively.

The results reported here lay the groundwork for determination of the TRAP three-dimensional structure by isomorphous replacement. Further work will include locating the 22 bromine atoms and subsequent 22-fold averaging of the initial electron density. The discovery that the TRAP molecule contains 11 subunits along with the recent indications that TRAP recognizes 11 U/GAG repeats in the *trp* leader (Babitzke & Yanofsky, 1994) suggests that each subunit binds one trinucleotide repeat.

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